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Stimulation of Vesicular-Arbuscular Mycorrhizal Fungi by Mycotrophic and Nonmycotrophic Plant Root Systems

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Transformed root cultures of three nonmycotrophic and one mycotrophic plant species stimulated germination and hyphal growth of the vesicular-arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerd.) in a gel medium. However, only roots of the mycotrophic species (carrot) supported continued hyphal exploration after 3 to 4 weeks and promoted appressoria formation by *G. etunicatum*.

The infection of roots by vesicular-arbuscular mycorrhizal (VAM) fungi involves a complex interaction among plant, soil, VAM fungi, and other soil microbes. While a number of reports have shown that soil extracts (4), host root exudates (3, 5, 6, 8–10), and, specifically, flavonoid compounds (1, 6, 10, 15) are stimulatory to VAM fungi, our knowledge concerning the mechanisms of communication between plant roots and mycorrhizal fungi is very incomplete. The recently successful dual sterile culture of VAM fungi with genetically transformed root systems (2) may be a useful tool in elucidating the chemical means of communication between the symbionts in VAM-plant interactions. In addition, a comparison of mycotrophic and nonmycotrophic plant species by using transformed roots may be useful to investigate the stages of VAM fungal development that are important in determining the compatibility between the host and fungus.

In this study, we compared the responses of the VAM fungus *Glomus etunicatum* (Becker & Gerd.) to the presence of transformed roots of a mycotrophic plant species and three nonmycotrophic plant species. Cultures of Ri T-DNA-transformed roots of carrot (*Daucus carota* L.) and sugar beet (*Beta vulgaris* L.) were kindly provided by Guillaume Becard (USDA Eastern Regional Research Laboratory, Philadelphia, Pa.). Transformed root cultures of *Brassica kaber* (DC) Wheeler. and *Brassica nigra* L. were prepared by inoculating sterile plants with *Agrobacterium rhizogenes* 15834. The *A. rhizogenes* culture was obtained from Hector Flores (Department of Plant Pathology, The Pennsylvania State University, University Park). Genetically transformed ("hairy") roots produced at inoculated wound sites were excised and transferred to a solid modified White's medium (by using Gel-rite; Scott Laboratories, Carson, Calif.; MW medium; see reference 2) containing 250 mg of carbenicillin liter^{-1}. The transformed roots were cultured in the dark at 25°C, and root tips that displayed rapid growth were transferred a second time to MW medium with carbenicillin. Only one clone of each of the root cultures produced was maintained on medium lacking antibiotic (MW medium) for later experiments so that all cultures of a given species were of the same genotype.

Hairy root cultures of *B. kaber*, *B. nigra*, beet, and carrot were subcultured for at least three generations on three separate media differing in the concentration of supplied sulfur but all with reduced phosphorus, sucrose, and sodium concentrations as suggested by Becard and Fortin (2) for dual culture with VAM fungi. Sulfur concentrations were manipulated in the media in the hope of reducing the quantity of glucosinolate-derived defensive compounds in the roots of the mustard species examined here (see references 12 and 13). Root tips of each of the plant species were placed on 7 to 10 plates each of three different media (HS medium, 3.03 mM SO_4^- [identical to M medium in reference 2]; LS1 medium, 1.03 mM SO_4^{-1}; LS2 medium, 0.53 mM SO_4^{-1}). Three days after the roots were transferred to the plates, approximately 20 surface-sterilized spores of *G. etunicatum* obtained from pot cultures on *Sorghum bicolor* L. were placed on the surface of the gel 1 to 2 cm in front of a growing root tip. Spores were also transferred to control plates of each medium without roots.

G. etunicatum spores were surface sterilized in 10% (vol/vol) bleach (0.524% sodium hypochlorite) containing 0.05% (vol/vol) Triton X-100 for 7 min and rinsed five times with sterile distilled water. Spores were aseptically transferred to sterile liquid HS medium and incubated for 48 h at room temperature. After 48 h, the spores were treated again with 10% bleach for 4.5 min, rinsed in sterile distilled water five times, and resuspended in sterile distilled water. Surface-sterilized spores were used immediately after the second bleach treatment.

The plates containing transformed roots and VAM fungal spores were placed in an incubator at 25°C. Humidified carbon dioxide (2%, balance air) was bled into the incubator from a tank. The concentration of CO_2 was monitored periodically inside the growth chamber with an infrared gas analyzer (model 306D; Nova Analytical Systems, Niagara Falls, N.Y.). The CO_2 level was maintained between 0.5 and 1.0%.

All of the plates were examined microscopically at weekly intervals for 4 weeks. Only those plates that were not contaminated by other microbes up to the fourth week were included in the analysis. The percentage of spore germination was determined, and hyphal lengths were estimated by using an ocular grid at a magnification of ×40. In addition, the behavior of VAM fungal hyphae as they approached the roots was carefully observed.

After 4 weeks, portions of roots from selected plates were cut out of the gel from approximately a 1-cm radius of the location of the VAM fungal spores. These small root pieces

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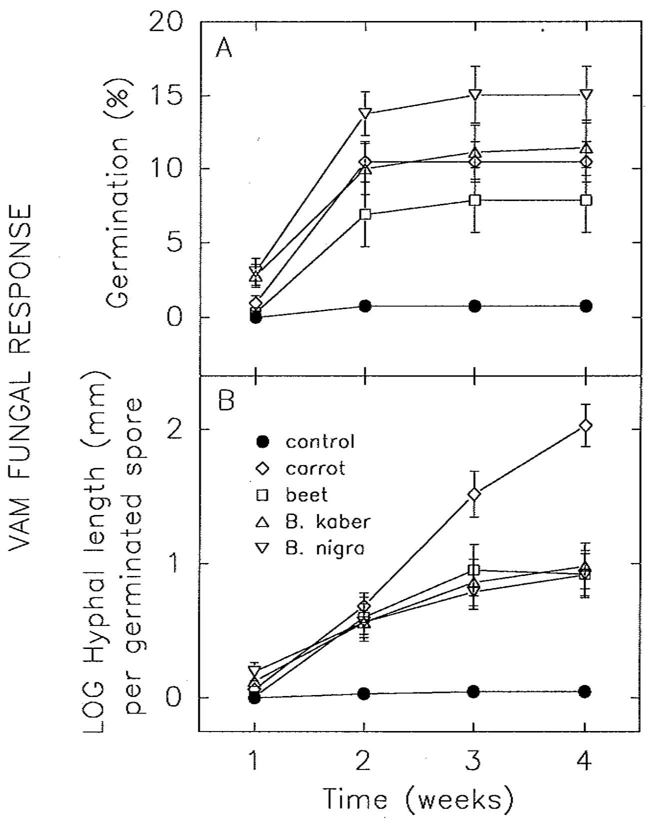

FIG. 1. Effects of transformed root cultures from four species on percentage of germination (A) and log (hyphal length [in millimeters] + 1) per germinated spore of *G. etunicatum* (B). Each point represents the mean of results on three media ± standard errors. (Analysis of variance significance values for germination; P = 0.0004, 0.0001, 0.0001, and 0.0001 at 1, 2, 3, and 4 weeks, respectively; for log [hyphal length + 1]; P = 0.0027, 0.0002, 0.0001, and 0.0001 at 1, 2, 3, and 4 weeks, respectively.)

were stained directly with trypan blue (0.1% in lactoglycerin, 25°C) overnight and examined under the microscope for the presence of hyphae on the rhizoplane and for appressoria formation. Both hyphal length on the rhizoplane and the number of appressoria were quantified for the root segments taken from each individual plate.

Statistical analysis on the measured VAM fungal variables was conducted by using SAS (Statistical Analysis System; SAS Institute, Inc., Cary, N.C.). Since the VAM fungal variables were not significantly ($P \leq 0.05$) affected by the media or the plant species by the medium interaction, a single-factor analysis of variance was constructed to examine the effects of plant species on VAM fungal spore germination and hyphal length at each weekly measurement period. (Note that the low-sulfur media used for this experiment (LS1 and LS2) and a third low-sulfur medium (LS3, 0.15 mM SO_4^{-1}) employed in a subsequent experiment also did not affect the quantity of glucosinolate-derived compounds in *B. kaber* root extracts; see reference 11.) Mean contrasts were constructed by using Fisher's protected least significant difference procedure at the 95% confidence level.

Spores of the VAM fungus *G. etunicatum* responded positively to the presence of transformed roots from all four species examined on solid medium (Fig. 1). Both spore germination and early hyphal extension were stimulated by the presence of all root cultures, irrespective of mycotrophic status, compared with the controls (no roots). However, roots from carrot supported significantly greater hyphal lengths after 3 and 4 weeks than any of the nonmycotrophic species (Fig. 1).

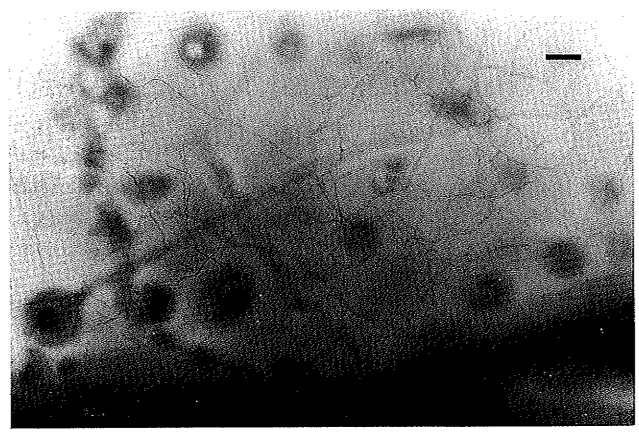

FIG. 2. Extensive hyphal network produced by *G. etunicatum* in the presence of transformed carrot roots after 4 weeks. Bar, 100 μm.

Spores that germinated on control plates produced only small amounts of hyphae extending a mean distance of only 0.14 mm per germinated spore by the fourth week. The mean hyphal lengths per germinated spore from plates with root cultures of *B. kaber*, *B. nigra*, and beet were 22.5, 11.3, and 15.0 mm, respectively. These values are comparable to the hyphal lengths reported for *G. etunicatum* in the presence of the VAM-stimulatory flavonol quercetin (1). The mean hyphal length per germinated spore for carrot plates was 228 mm.

Hyphae that approached the roots of carrot often branched extensively within 100 to 1,000 μm of the root surface (Fig. 2). Hyphae rarely crossed over the roots of carrot and appeared to grow on the surface of the roots since hyphae could be seen coming away from the root surface farther along the root axis. Large, highly branched hyphal networks occurred near the roots of carrot, sometimes expanding to a measured diameter of 15 mm. Networks like the ones observed near carrot roots were not observed near the roots of any of the other species examined. VAM hyphae growing on the plates of nonmycotrophic root cultures were much less branched, and the hyphae tended to elongate along relatively straight lines.

Germ tubes often crossed right over the roots of the nonmycotrophs (*B. kaber*, *B. nigra*, and beet) and continued to elongate without altering their direction or branching. In some cases, however, the hyphae did branch once or twice either before encountering or after crossing the root of a nonmycotrophic species. Germ tubes that came within 50 to 100 μm of the roots of either mustard species often ceased to elongate, and the cytoplasm within the hyphae retracted from the growing tip (Fig. 3, *B. nigra* root). Retracted cytoplasm within hyphae was never observed whenever germ tubes came within very close proximity of the roots of the mycotroph carrot.

The analysis of the presence of external VAM structures on stained roots showed that hyphae and numerous appressoria structures occurred on the roots of carrot (Table 1). No appressoria were found on the roots of the nonmycotrophic

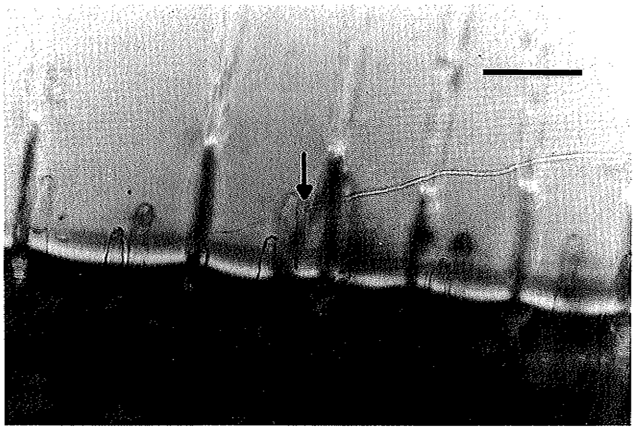

FIG. 3. Hyphal tip from a germinated spore of *G. etunicatum* showing retracted cytoplasm (arrow) after a close encounter with the surface of transformed *B. nigra* root. Bar, 100 μm.

species. A mean value of 4.1 appressoria per plate was found on the carrot roots.

The fact that all of the species examined here were capable of stimulating VAM fungal spore germination and early hyphal growth indicates that the stimulatory signal(s) responsible for this first step in VAM fungal development from resting spores is relatively nonspecific. However, only roots of the mycotrophic species carrot resulted in VAM hyphal growth on the rhizoplane and appressoria formation, suggesting that a different signal(s) is required for the development of the fungus on the root itself. In fact, it appeared that carrot roots produced spatial information that was perceived by the fungus while the nonmycotrophic roots did not. For example, whenever hyphae approached a carrot root that was below the surface of the gel, the hyphae branched and explored the surface of the gel directly above the underlying root axis in both directions. This was not observed whenever hyphae approached submerged roots from any of the nonmycotrophs.

While there was not a difference in the abilities of the nonmycotrophs and the host carrot to stimulate the germination and early hyphal growth of *G. etunicatum*, the nonmycotrophs did not attract the hyphal tips to the root surface. It appears, therefore, that the nonmycotrophs possess the correct signal(s) to stimulate the germination and early hyphal growth of *G. etunicatum* but possibly do not produce the correct signal(s) involved in later stages of VAM-plant interactions. However, the involvement of inhibitory compounds in the rhizoplane interactions of *G. etunicatum* with the nonmycotrophs reported here cannot be ruled out, especially in light of the observations here and elsewhere (7, 14) of retracted cytoplasm in VAM hyphal tips that have come within close proximity of mustard roots.

TABLE 1. External VAM structures on selected samples of transformed roots

Species	n	Total no. of appressoria	Mean no. of appressoria per plate	Hyphal length (mm)/cm of root
Carrot	9	37	4.1	1.774
Beet	7	0	0	0.014
B. kaber	5	0	0	0.017
B. nigra	3	0	0	0

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